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Pharmacologically Triggered Hydrogel for Scheduling Hepatitis B Vaccine Administration

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Received
7 May 2013

Accepted
12 August 2013

Published
10 September 2013

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The simplification of current vaccine administration regimes is of crucial interest in order to further sustain and expand the high impact of vaccines for public health. Most vaccines including the vaccine against hepatitis B need several doses to achieve protective immunization. In order to reduce the amount of repetitive injections, depot-based approaches represent a promising strategy. We present the application of novobiocin-sensitive biohybrid hydrogels as a depot for the pharmacologically controlled release of a vaccine against hepatitis B. Upon subcutaneous implantation of the vaccine depot into mice, we were able to release the vaccine by the oral administration of the stimulus molecule novobiocin resulting in successful immunization of the mice. This material-based vaccination regime holds high promises to replace classical vaccine injections conducted by medical personnel by the simple oral uptake of the stimulus thereby solving a major obstacle in increasing hepatitis B vaccination coverage.

Routine vaccination is one of the most effective means to increase global health with the power to completely eliminate infectious diseases such as smallpox¹ and polio². In order to increase worldwide immunization, vaccines are actively promoted by large vaccination campaigns such as the Global Alliance for Vaccines and Immunization (GAVI)³ having prevented 5.4 million future deaths in 10 years⁴. However, these campaigns are accompanied with huge financial (US\$ 4.5 billion in 10 years for GAVI), organizational and logistic efforts due to the fact that repeated vaccine injections associated with several medical consultations are needed. As the density of health workers is a major obstacle to achieve sufficient vaccination coverage⁵, novel vaccine development and delivery technologies reducing professional medical care are estimated to have a major future impact on health in developing countries⁶. This is especially a problem in the case of hepatitis B virus (HBV) infections with 600,000 deaths per year⁷ although the worldwide coverage with the respective vaccine is estimated at 75%⁸. One promising strategy to reduce medical consultations associated with the prime-boost vaccination regime would be the replacement of repeated injections by the release of the vaccine from a body depot. As the prime and boost vaccine doses need to be released to the body in a pulse-like profile in order to induce long-lasting immune protection, stimulus-sensitive materials⁹ would be well suited to act as such a depot as they would allow for the development of a release profile similar to the conventional vaccine delivery.

With the development of biohybrid materials being responsive to clinically approved small molecules at pharmacologically feasible concentrations^{10–14}, a promising direction towards a safe and patient-compliant biomedical use of stimulus-inducible hydrogels was discussed. To this aim, branched polymers were functionalized with protein domains such as the gyrase B (GyrB) thereby allowing for the coumermycin-inducible dimerization of the GyrB moieties resulting in crosslinking of the polymers to a hydrogel^{10,15}. Finally, the dissolution of this biohybrid material was achieved in a stimulus-inducible manner by the addition of novobiocin.

In this study we developed a novel strategy to reduce repetitive hepatitis B vaccine injections by the release of the vaccine from a body depot in response to an orally available stimulus. To this aim, we incorporated a commercially available hepatitis B vaccine (Engerix-B¹⁶) into a PEG-based biohybrid hydrogel depot being responsive to the clinically licensed molecule novobiocin. Upon implantation into mice, the depot was dissolved

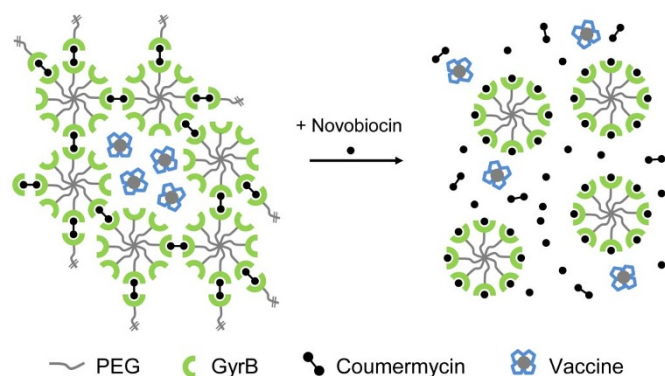


Figure 1 | Design of the drug-responsive hydrogel vaccine depot. A molecular switch based on the interaction of the protein Gyrase B (GyrB) to the aminocoumarin antibiotics coumermycin and novobiocin is providing the responsiveness to the hydrogel. To this aim, 8-arm polyethylene glycol (PEG) was end-functionalized with GyrB. Addition of coumermycin dimerized GyrB thereby leading to hydrogel formation and physical entrapment of the vaccine. Addition of novobiocin competitively replaced coumermycin resulting in GyrB monomerization and hydrogel dissolution and the release of the vaccine.

at the scheduled point in time by the oral administration of the stimulus thereby triggering the release of the vaccine. The elicited immune response was comparable to conventional repetitive vaccine injections. This orally controlled vaccination regime holds high promises for the simplification of hepatitis B vaccine delivery by reducing the repetitive medical consultations thereby solving a major obstacle in increasing hepatitis B vaccination coverage.

Results

PEG-based pharmacologically tunable vaccine depot. As material for the storage and the inducible release of the commercial hepatitis B vaccine Engerix-B, we synthesized a polyethylene glycol (PEG)-based hydrogel which incorporates the GyrB-aminocoumarin switch¹⁵ rendering the material stimulus-responsive (Fig. 1). To this aim, branched PEG molecules were covalently decorated with the N-terminal subunit of the protein Gyrase B (GyrB). Addition of the aminocoumarin antibiotic coumermycin leads to the dimerization of the GyrB moieties resulting in hydrogel formation. Due to its large size (from 100 nm up to 10 μm ¹⁷), the vaccine can physically be incorporated into the hydrogel network structure. Addition of the inducing stimulus novobiocin competitively replaces coumermycin from the GyrB protein resulting in hydrogel dissolution and the release of the incorporated vaccine.

The GyrB protein was expressed in *E. coli* with a C-terminal hexahistidine tag for purification and a cysteine for the coupling to the PEG (GyrB-Cys). In order to further stabilize the hydrogel by additional crosslinks, a GyrB variant harboring an N- and C-terminal cysteine was produced (Cys-GyrB-Cys). For the formation of the hydrogel, the GyrB proteins were mixed with coumermycin at a molar ratio of 2 : 1 followed by the coupling via its cysteines to vinyl-sulfone-modified 8-arm PEG (PEG-VS, molar ratio of GyrB to VS groups of 1 : 1) by a Michael-type addition mechanism. Initial tests with different molar ratios of the cysteine-modified protein variants revealed high inducibility of the hydrogel with a molar ratio of GyrB-Cys to Cys-GyrB-Cys of 4 : 1 (Supplementary Fig. 1). This resulted in a typical hydrogel composition of 401 μg (16 nmol) GyrB-Cys, 101 μg (4 nmol) Cys-GyrB-Cys, 11.1 μg (10 nmol) coumermycin and 93.8 μg (20 nmol VS groups) PEG-VS in a hydrogel volume of 5 μl . The chemical structure of the synthesized hydrogel was further analyzed by NMR thereby confirming the quantitative reaction of the polymer with the protein (Supplementary Fig. 2).

Having developed the hydrogel depot prototype, we evaluated its potential to function as hepatitis B vaccine depot. To this aim, commercially available hepatitis B vaccine (Engerix-B, GlaxoSmith-Kline¹⁶) being composed of recombinantly expressed hepatitis B surface antigen (HBsAg) particles adsorbed to metalhydroxide particles (alum) were added to the synthesis during hydrogel formation leading to 1 μg HBsAg content per 5 μl hydrogel. Transfer of the formed hydrogel depots to PBS resulted in 25% volumetric swelling (Supplementary Fig. 3). During this period, only marginal amounts of vaccine were released summing up to 4.8 (± 5) ng released vaccine after 1 week.

The viscoelastic properties of the hydrogel vaccine depot were analyzed by small-strain oscillatory shear rheometry in a frequency range of 0.1 to 1 Hz resulting in storage moduli (G') largely exceeding the loss moduli (G'') (Fig. 2a). These typical values for a viscoelastic polymer network are in congruence with published data¹⁵ and demonstrate that the vaccine components do not negatively affect the mechanical properties of the hydrogel.

Hydrogel vaccine release characteristics. For an application as vaccine depot *in vivo*, the material needs to be sensitive to its inducer under physiological conditions leading to the dissolution of the hydrogel and the release of the incorporated vaccine load. We tested these aspects *in vitro* by the incubation of vaccine-loaded hydrogels in PBS containing 100 μM novobiocin and monitoring the dissolution and release of the hydrogel protein components by Bradford assay (Fig. 2b). Whereas hydrogels incubated in the absence of novobiocin only showed a marginal release of hydrogel components reaching a plateau level of 5% dissolution after 8 h, addition of 100 μM novobiocin led to the rapid and complete dissolution of the hydrogel within 2 h. Simultaneously to the dissolution of the hydrogel, the incorporated vaccine was also released with similar kinetics in the presence of novobiocin whereas the absence of the inducer led to no detectable vaccine release as determined by the quantification of the HBsAg content in the supernatant (Fig. 2c).

In order to test the hydrogel's suitability for the release of vaccines in the body, we simulated the body distribution kinetics of orally administered novobiocin by novobiocin pulse experiments (Fig. 2d). Whereas no release of vaccine was detected with pulses up to 10 min and partial release was observed with 20 min pulses of 100 μM novobiocin, complete dissolution of the depot and release of the vaccine was achieved when novobiocin was present for 1 h. This confirms the suitability of the depot for applications *in vivo* as such a pulse profile necessary for the response time of the depot can be achieved in mice^{18,19} as well as in humans^{20,21} without affecting biocompatibility.

In order to test the depot for the longtime storage and release of vaccines in the body, we incubated vaccine-loaded hydrogels at 4 °C and 37 °C in synthetic body fluid (SBF²²) emulating the ionic composition of extracellular body fluids. After 1 week, the hydrogels stored at both temperatures still retained their responsiveness as tested by the incubation in the presence of 100 μM novobiocin (Fig. 2e) thereby releasing equal amounts of vaccine as compared to fresh hydrogel depots (Fig. 2c).

Pharmacologically scheduled vaccination in mice. Encouraged by the positive *in vitro* results regarding the suitability of the PEG-based hydrogel as a vaccine depot, we evaluated its application as a depot for the stimulus-inducible hepatitis B vaccine release for immunization in mice (Fig. 3). This strategy would allow replacing the boost injection of the vaccine by the simple oral administration of the stimulus molecule thereby drastically reducing the need for repetitive medical consultations. To this aim, we vaccinated mice with a prime vaccine dose of the hepatitis B surface antigen (HBsAg) vaccine (0.5 μg HBsAg per mouse) and co-administered a vaccine-loaded hydrogel (releasing 0.5 μg HBsAg as quantified in

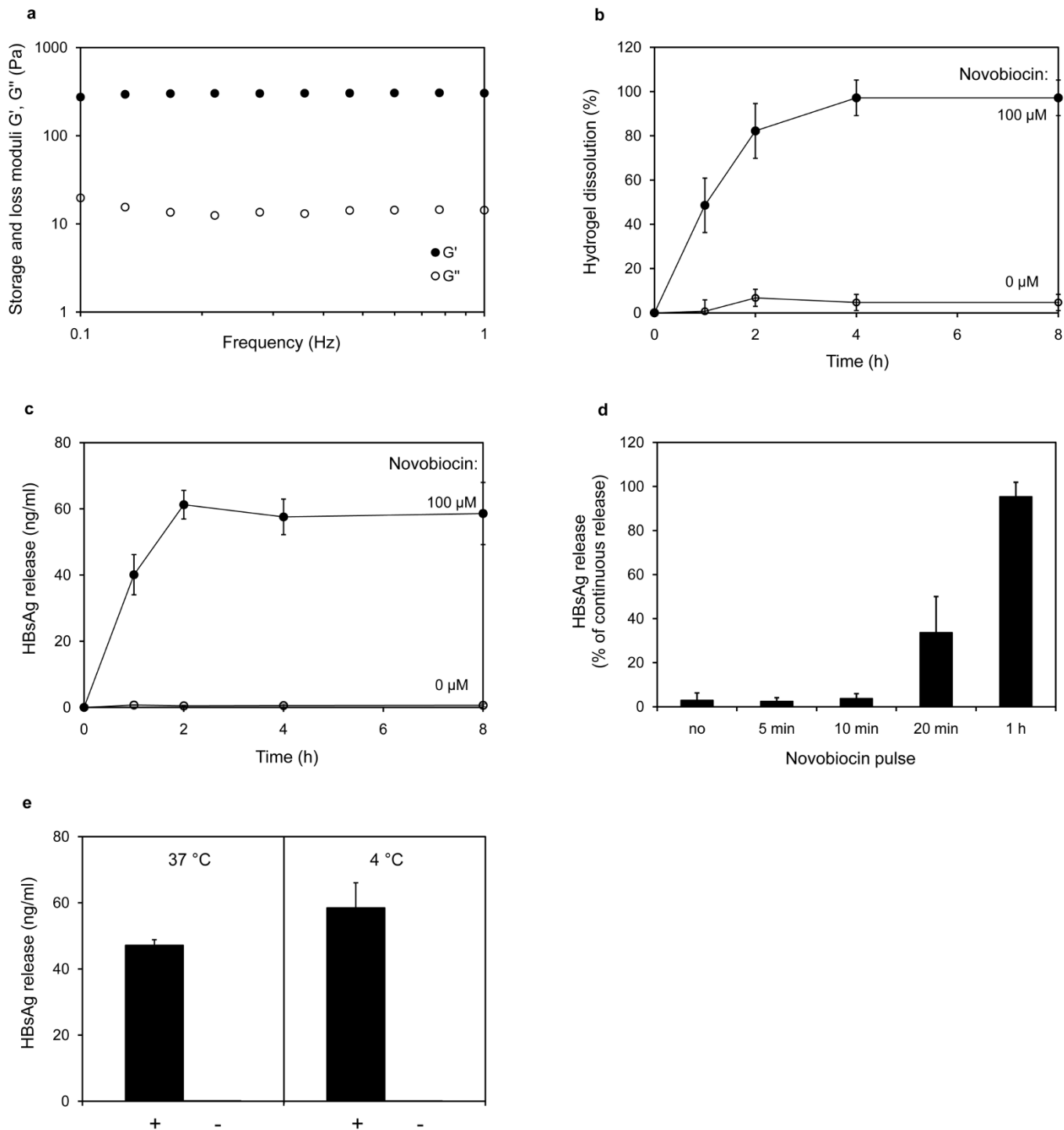


Figure 2 | Hydrogel vaccine depot characterization. (a) Viscoelastic properties of the hydrogel vaccine depot. Hydrogel discs of 40 μ l harboring the hepatitis B vaccine were prepared and pre-swollen in PBS for 1 h. The storage and loss moduli G' and G'' were determined by small-strain shear rheometry at frequencies from 0.1 to 1 Hz. (b) Novobiocin-inducible hydrogel dissolution. Hydrogels (5 μ l) harboring the commercial hepatitis B vaccine (Engerix-B) were incubated in PBS in the absence or presence of 100 μ M novobiocin. Hydrogel dissolution was monitored by the quantification of the released hydrogel protein components into the supernatant. Error bars represent the standard deviation of 6 replicas. (c) Novobiocin-inducible vaccine release. 5 μ l hydrogels harboring the hepatitis B vaccine (0.2 μ g/ μ l HBsAg content) were incubated in PBS optionally containing 100 μ M novobiocin. The release of the vaccine from the hydrogels was followed by the quantification of the HBsAg content in the supernatant. Error bars represent the standard deviation of 4 replicas. (d) Novobiocin pulse-dependent vaccine release. 5 μ l hydrogels harboring the hepatitis B vaccine (0.2 μ g/ μ l HBsAg content) were incubated in PBS containing 100 μ M novobiocin for the indicated time of the pulse followed by washing and further transfer to fresh PBS for 4 h. For samples without novobiocin pulse (no), hydrogel depots were incubated in PBS for 1 h prior to transfer to fresh PBS for 4 h. The release of the vaccine from the hydrogels was analyzed by the quantification of the total HBsAg content in the supernatant and expressed in % of the total continuous release from (c). Error bars represent the standard deviation of 4 replicas. (e) Long-term functionality of the hydrogel vaccine depot. 5 μ l hydrogels containing the hepatitis B vaccine (0.2 μ g/ μ l HBsAg content) were incubated in synthetic body fluid (SBF) at 4 °C and 37 °C for 1 week. Afterwards, hydrogels were transferred to fresh SBF supplemented with 100 μ M (+) or without (−) novobiocin. The release of the vaccine from the hydrogel was monitored by the quantification of the HBsAg content in the supernatant. Error bars represent the standard deviation of 3 replicas.

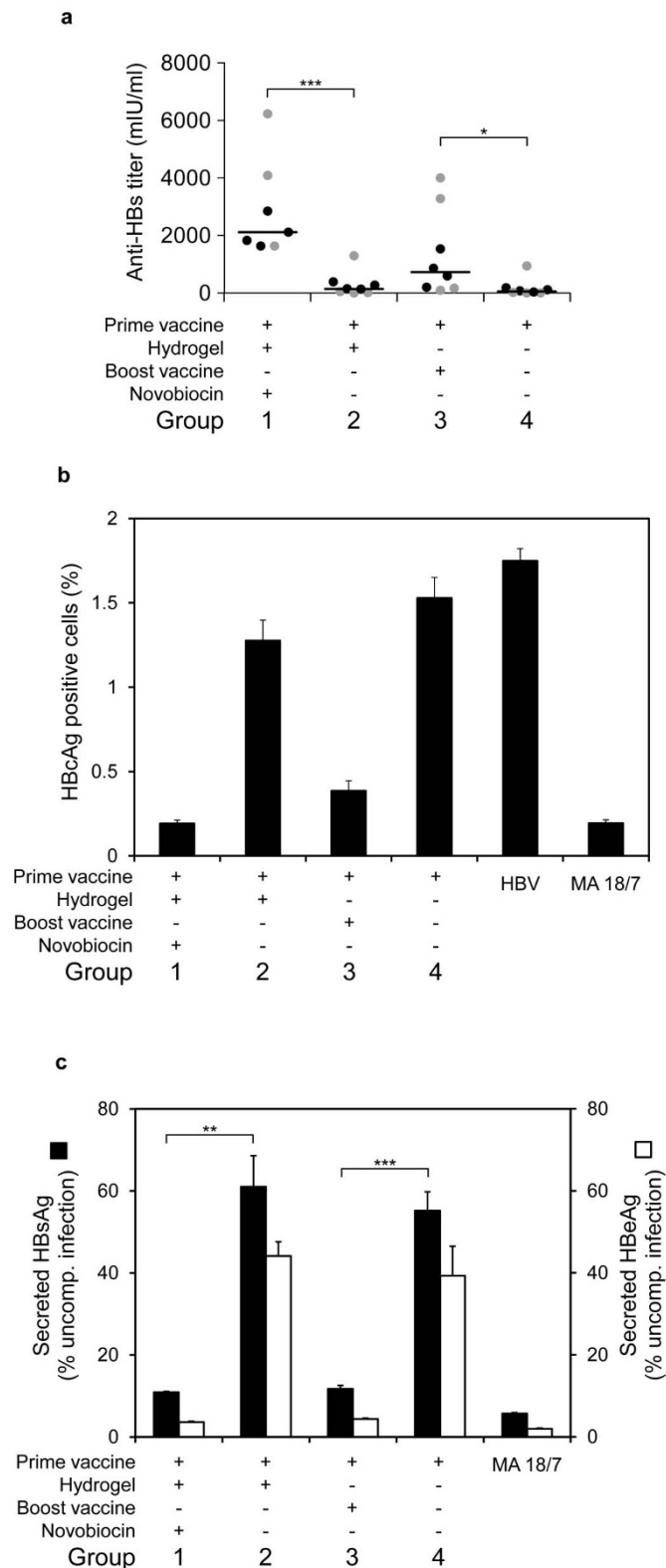


Figure 3 | Time-scheduled hydrogel-mediated hepatitis B vaccination in mice. (a) Remote-controlled vaccination with hydrogels. A prime vaccine dose (0.5 μ g HBsAg) was administered to all mice on day 1. A hydrogel of 50 μ l harboring 0.5 μ g HBsAg vaccine was subcutaneously administered to the mice of groups 1 and 2 on day 1. Oral doses of novobiocin were administered to mice of group 1 twice on day 6 (15 and 11.25 mg per mouse) and once on day 7 and 8 (15 mg per mouse). The boost vaccine dose (0.5 μ g HBsAg) was administered to group 3 on day 7. At day 98, serum was withdrawn and HBsAg-specific antibody titers (anti-HBs) were

determined. The median of the anti-HBs titers is shown. Sera used for the neutralization experiments are highlighted in black. (b) Hepatitis B virus (HBV) neutralization activity of mouse sera. The sera of 4 mice with an anti-HBs titer around the median of each group from (a) were pooled and pre-incubated (in triplicates) with HBV followed by infection of HepaRG cells and further incubation. On day 12 post-infection (p.i.), the percentage of positive cells for the HBV core antigen (HBcAg) was determined by HBcAg-specific immune fluorescence staining in comparison to the total cell number (DAPI stain). As an uncompleted control, HBV pre-incubated without sera (HBV) and as a positive control, HBV pre-incubated with a neutralizing antibody (MA 18/7) was used. (c) Quantification of secreted viral markers. The amount of HBsAg and HBeAg (hepatitis B e antigen) secreted from day 8 to 12 p.i. during the neutralization experiment in (b) was determined by ELISA. The values are given as percentage of the uncompleted (uncomp.) infection. Values of Secreted HBsAg were analyzed for statistical significance. The sensitivity of the ELISA (cutoff) is 0.25% for HBsAg and 0.5% for HBeAg, respectively. Error bars represent the standard error of the mean. * indicates differences in the median at a level of significance with $p < 0.05$; ** corresponds to $p < 0.01$ and *** to $p < 0.001$.

Fig. 2b) subcutaneously on day 1. In order to dissolve the hydrogel depot and release the boost vaccine dose, we orally administered the stimulus molecule novobiocin on day 6, 7 and 8 (group 1, Fig. 3a). For the stability evaluation of the vaccine depot in the body, we kept a group of vaccine depot-harboring mice without administration of novobiocin. For comparison to the classical immunization regime, we injected to mouse groups the prime and boost vaccine doses on day 1 and 7 or only a prime dose on day 1 (0.5 μ g per dose, groups 3 and 4, respectively, Fig. 3a). After 98 days, the protective immune response was determined by the quantification of the HBsAg-specific antibody titer (anti-HBs, Fig. 3a). The mice harboring a vaccine depot and having received the novobiocin stimulus (group 1) showed significantly higher anti-HBs titers as compared to mice not having received novobiocin (group 2) thereby demonstrating that the vaccine is released from the depot in a stimulus-inducible manner. Comparison of the hydrogel-mediated (group 1) to the classical 2-dose injection regime (group 3) showed similar anti-HBs titer levels demonstrating the efficacy of the novel depot-based immunization. Additionally, mice not supplemented with the stimulus novobiocin (group 2) retained the vaccine efficiently in their hydrogel depot resulting in anti-HBs titers comparable to the mice having only received a single prime vaccine dose (group 4).

In addition to the determination of anti-HBs titers, we characterized the ability of the sera to inhibit HBV infection in a cell-based neutralization assay (Fig. 3b,c). To this aim, we pooled the sera of 4 mice of each respective group showing an anti-HBs titer around the median (highlighted in black in Fig. 3a). For the neutralization

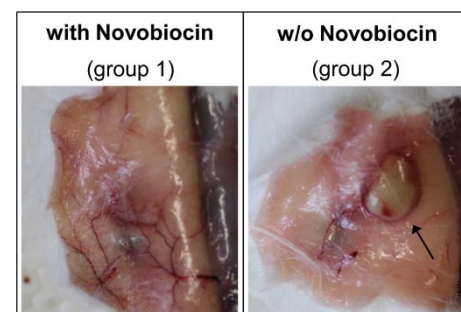


Figure 4 | Macroscopic characterization of the vaccine hydrogel depot. Mice of group 1 and 2 (see Fig. 3a) were sacrificed at day 98 and the site of gel administration was analyzed. The remaining non-dissolved hydrogel of the mouse of group 2 is indicated by an arrow.



experiment, the pooled sera were pre-incubated with a HBV inoculum and subsequently tested for their infectivity to differentiated HepaRG cells²³. The extent of HBV neutralization was determined by the amount of HepaRG cells positive for the hepatitis B core antigen (HBcAg) (Fig. 3b) as well as by the quantification of the cell-secreted viral antigens HBsAg and hepatitis B e antigen (HBeAg) (Fig. 3c). The hydrogel-mediated (group 1) and the classical (group 3) 2-dose regime showed high neutralization efficacy resulting in low amounts of HBcAg positive cells and secreted HBsAg/HBeAg similar to the pre-S1 specific HBV neutralizing antibody MA 18/7^{24,25}. On the other hand, the sera of mice non-fed with novobiocin (group 2) only showed a rudimentary protective effect being comparable to the mice only having received a single vaccine dose (group 4). These observations are in line with the levels of anti-HBs titers and further underline comparable efficacy of the hydrogel-mediated and the classical injection regime.

The promising immunological results were also reflected by the macroscopic analysis of the hydrogel depot of mice sacrificed after 98 days (Fig. 4). Whereas inspection of the vaccine depot administration site of the mice lacking novobiocin treatment showed an intact tissue-surrounded hydrogel (arrow), no subcutaneous remnants of the hydrogel were observed in mice treated with novobiocin.

Discussion

In this study, we presented a novel depot-based strategy for the on-command scheduled delivery of a hepatitis B vaccine in mice. With the developed hydrogel depot being sensitive to the pharmaceutical substance novobiocin, the repetitive delivery of a vaccine could be drastically simplified by the simple oral intake of a tablet containing the stimulus molecule. In contrast to other innovative polymer-based vaccination methods^{26,27} and remote-controlled delivery strategies such as implanted microchips which have recently been shown to deliver therapeutic proteins in a pulse-like fashion in human²⁸, our system based on an orally available stimulus allows for simple handling and therefore represents a highly attractive alternative for the simplification of vaccination regimes especially in developing countries. This hydrogel depot may further be suitable for the externally controlled delivery of numerous other vaccines and biopharmaceuticals.

Methods

Protein production. The vector pRG053 for the bacterial expression of Cys-GyrB-Cys under the control of the phage T7 promoter was constructed by the amplification of the 5'-part of the gyrB gene from chromosomal *E. coli* K12 DNA using oligonucleotides ORG033 (5'-gtatcacatgatgtctcgattcttactctccag-3') and OWW985 (5'-ccagttacaagcttcagcaatgtgatgtgatgtgcttcattggaagtgtcttc-3') and cloning the PCR product NdeI/HindIII into pWW301²⁹.

The vectors pRG053 and pWW1166¹⁵ for the expression of Cys-GyrB-Cys and GyrB-Cys were transformed into *E. coli* BL21 STAR™ (DE3) (Life Technologies, Carlsbad, CA, cat. no. C601003). Protein production was induced in LB medium at OD₆₀₀ = 0.8 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. The cells were pelleted (6000 × g, 7 min, 4°C), resuspended in lysis buffer (40 ml per 1000 ml initial culture volume, 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted using a French press (1000 bar, 3 passages, APV, DK, Albertslund, APV-2000). Cell debris was eliminated (30,000 × g, 30 min, 4°C) and the lysate was loaded onto a gravity flow Ni²⁺-NTA-agarose Superflow column (10 ml lysate per ml Ni²⁺-NTA-agarose bead volume, Qiagen, Hilden, Germany, cat. no. 30210) followed with washing with 10 column volumes lysis buffer, 10 column volumes wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and elution with 2 column volumes elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). To the eluate, 10 mM ethylenediaminetetraacetic acid sodium salt, pH 8 (EDTA) was added and stored at -80°C. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, cat. no. 500-0006) with bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO, cat. no. 05479) as standard.

Hydrogel synthesis. The proteins Cys-GyrB-Cys and GyrB-Cys were concentrated to 100 mg/ml by ultrafiltration (10 kDa MWCO, Corning, Lowell, MA, cat. no. 431483), a 20-fold molar excess of TCEP (Tris(2-carboxyethyl)phosphine hydrochloride, Sigma Aldrich, cat. no. C4706, 33 mg/ml in 500 mM NaHCO₃ buffer pH 9) was added and incubated at room temperature for 1 h. The buffer of the reduced proteins was exchanged to reaction buffer (2.68 mM KCl, 1.47 mM

KH₂PO₄, 8.03 mM Na₂HPO₄, 137 mM NaCl, 2 mM EDTA pH 8) on a desalting column (Thermo Fisher Scientific, Waltham, MA, cat. no. 43233) and the protein was concentrated to 200 mg/ml by ultrafiltration under nitrogen atmosphere.

Typical 5 μl hydrogels were prepared by mixing the proteins GyrB-Cys (401 μg, 16 nmol) and Cys-GyrB-Cys (101 μg, 4 nmol) in a molar ratio of 4:1 (unless indicated otherwise) to a total amount of 20 nmol protein in 4 μl reaction buffer followed by the addition of coumermycin (Promega, Madison, WI, cat. no. C9451, 50 mg/ml in DMSO) in a molar ratio of GyrB:coumermycin = 2:1 and incubation at room temperature for 1 h. Hydrogel formation was started by the addition of 0.15 μl of 3.3 M triethanolamine and 93.8 μg (20 nmol VS groups) 8-arm polyethyleneglycol-vinylsulfone (PEG-VS, derived from a previous study¹⁵). Hydrogel formation was completed by incubation at 25°C in a humidified atmosphere for 24 h.

For the production of vaccine-loaded hydrogels, commercially available hepatitis B vaccine (Engerix-B, GlaxoSmithKline, München, Germany, cat. no. 7504643, 20 μg/ml Alum-adsorbed HBsAg) was concentrated to 0.4 mg/ml by centrifugation (13,000 × g, 1 min). For hydrogel formation, 0.2 μg/μl (final hydrogel volume) of concentrated HBsAg vaccine was added to the hydrogel prior to the addition of triethanolamine and PEG-VS.

Mechanical hydrogel characterization. For rheological analysis, vaccine-loaded hydrogel discs of 40 μl volume were prepared between two siliconized glass slides (1 mm height) and pre-swollen in PBS (2.68 mM KCl, 1.47 mM KH₂PO₄, 8.03 mM Na₂HPO₄, 137 mM NaCl, pH 7.4) for 1 h. The storage and loss moduli (G' and G'') were determined by small strain oscillatory shear experiments on a modular advanced rheometry system II (MARS, Thermo Fisher Scientific) with parallel steel plates (20 mm) at 20°C. Hydrogels were placed between the plates and the gap was adjusted to 0.5 mm. Frequency-sweep experiments were performed at constant strain (20%) from 0.1 to 1 Hz.

Hydrogel characterization. For hydrogel dissolution kinetics, 5 μl vaccine-loaded hydrogels were pre-swollen in PBS for 24 h. Afterwards, they were transferred to 1 ml PBS containing the indicated novobiocin concentrations. The stimulus-responsive dissolution of the hydrogel was monitored by the quantification of the released protein components in the supernatant by the Bradford method. Released hepatitis B vaccine was quantified by ELISA (Enzygnost HBsAg 6.0, Siemens, München, Germany, cat. no. OPFM03) using fresh hepatitis B vaccine as a standard.

For novobiocin pulse-dependent vaccine release experiments, 5 μl vaccine-loaded hydrogels were pre-swollen in PBS for 24 h. Afterwards, they were incubated in 1 ml PBS containing 100 μM novobiocin for the corresponding time of the pulse followed by washing steps (4 × 2 min in 1 ml PBS) and further incubation in 1 ml fresh PBS for 4 h. For samples without novobiocin pulse, hydrogel depots were incubated in PBS for 1 h prior to washing and transfer to fresh PBS for 4 h. Total released hepatitis B vaccine was quantified by ELISA as mentioned above.

For long-term functionality tests, 5 μl vaccine-loaded hydrogels were transferred to 1 ml synthetic body fluid (SBF²²) supplemented with gentamycin (40 μg/ml) and puromycin (10 μg/ml) and incubated for 1 week at 4°C and 37°C, respectively. Afterwards, hydrogels were transferred to fresh SBF containing the indicated novobiocin concentrations and the released hepatitis B vaccine was quantified by ELISA as mentioned above.

Mouse experiments. For vaccination studies, female Swiss CD 1 mice (Janvier SAS C574105, Le Genest St Isle F-53941 St Berthevin, France) were used. A minimum amount of 7 mice was kept for each group. For standard vaccination, the commercial Engerix-B vaccine was diluted to 5 μg/ml in PBS and injected (100 μl per mouse). 50 μl hydrogels containing 0.2 μg/μl vaccine were applied subcutaneously on the back of the mice under sodium pentobarbital (Nembutoal, 45 mg/kg). Novobiocin was administered by gastric tube (75 mg/ml in 150 mM NaCl) in 2 doses (600 and 350 mg/kg) on day 6 and in 1 dose (600 mg/kg) on day 7 and 8. Serum samples were withdrawn on day 98. All animal experiments were performed according to the directives of the European Community Council (86/609/EEC), approved by the French Republic (no. 69266310), and performed by M.D.E. and G.C.E. at Lyon University.

Quantification of serum antibody levels. For the determination of total antibody to Hepatitis B surface antigen (anti-HBs), the mouse serum was analyzed using a sandwich immunoassay (ADVIA Centaur aHBs II, Siemens Healthcare Diagnostics, Eschborn, Germany) on an ADVIA Centaur XP system. Antibody titers were quantified in mIU/ml.

Neutralization experiments. Neutralization assays were performed as recently described²³. Briefly, HepaRG cells were grown in Williams' medium E supplemented with 10% heat-inactivated fetal calf serum, 50 U penicillin/ml, 50 μg streptomycin/ml, 5 μg insulin/ml, and 50 μM hydrocortisone hemisuccinate. The cells were split every 2 weeks at a ratio of 1:5. Fourteen days before infection, cell differentiation was induced by adding 1.5% DMSO to the maintenance medium³⁰. The medium was exchanged every 3 days.

HBV was produced in HepAd38 cells³¹ grown on 5-stack cell factories (Corning, cat. no. 3319). 450 ml supernatants were collected every third day and subjected to automated heparin affinity chromatography on 5 ml HiTrap Heparin columns (GE Healthcare, Piscataway, NJ, cat. no. 17-0407-01). Bound virions were eluted with a linear NaCl gradient. Immediately after elution, the virus peak fractions (340 ± 100 mM NaCl, 20 mM TrisCl, pH 7.4) were rebuffered to physiological



salt concentrations (140 mM NaCl) by addition of H₂O and supplemented with FCS to a final concentration of 5%. Aliquots were stored at −80°C.

For HBV infection, differentiated HepaRG cells (5×10^5 cells/well of a 24-well plate) were incubated with a 33-fold dilution of the concentrated HBV stock in medium in presence of 4% polyethylene glycol 8000 for 16 h at 37°C. For neutralization of HBV viral particles, the sera of 4 mice of each group with an antibody titer around the median (Fig. 3a) were pooled and pre-incubated with the HBV inoculum (3 µl serum were added per 7.5 µl diluted inoculum) for 30 min at 37°C, followed by co-incubation with the cells for 16 h at 37°C as described in²³. Pooled sera from mice taken before vaccination used in same volumes served as serum controls. The humanized monoclonal antibody MA 18/7 (derived from Humabs, Bellinzona, Switzerland), directed against the pre-S1 domain of the large HBs protein^{24,25} was used as a positive control (1 µg/ml). At the end of the incubation, the cells were washed three times and further cultivated. Medium was exchanged every 2 to 4 days. To guarantee the best compatibility between the different serum pools, the respective test was performed in 24-well plates with the same batch of cells. The amounts of hepatitis B surface antigen (HBsAg) and the hepatitis B e antigen (HBeAg) secreted into the supernatant from day 8 to 12 post-infection (p.i.) were determined by immunoassays (ARCHITECT HBsAg, Abbott Laboratories, Wiesbaden, Germany and ADVIA Centaur HBeAg, Siemens).

The HBV core antigen (HBcAg) as an additional virological marker was analyzed at day 12 p.i. by HBcAg-specific immunofluorescence as described in²³. Analysis of images was performed with ImageJ software using the nucleus counter function of the Wright Cell Imaging Facility (WCIF) plug-in to count the number of labeled nuclei (DAPI) and HBcAg-positive cells. For each serum pool, 10 images per well were analyzed.

Statistical analysis. Statistical comparison among groups was performed using a two-tailed unpaired t-test. p values ≤ 0.05 were considered statistically significant.

- Arita, I. Virological Evidence for the Success of the Smallpox Eradication Program. *Nature* **279**, 293–298 (1979).
- Hull, H. F. Pax Polio. *Science* **275**, 40–41 (1997).
- Nossal, G. J. V. The Global Alliance for Vaccines and Immunization—a millennial challenge. *Nat Immunol* **1**, 5–8 (2000).
- Nossal, G. J. Global immunization for the 21st century. *Science* **284**, 587 (1999).
- Anand, S. & Barnighausen, T. Health workers and vaccination coverage in developing countries: an econometric analysis. *Lancet* **369**, 1277–1285 (2007).
- Daar, A. S. *et al.* Top ten biotechnologies for improving health in developing countries. *Nat Genet* **32**, 229–232 (2002).
- Goldstein, S. T. *et al.* A mathematical model to estimate global hepatitis B disease burden and vaccination impact. *Int J Epidemiol* **34**, 1329–1339 (2005).
- Goldstein, S. Global Routine Vaccination Coverage, 2010 (Reprinted from MMWR, vol 60, pg 1520–1522, 2011). *Jama-J Am Med Assoc* **306**, 2662–2664 (2011).
- Mohammed, J. S. & Murphy, W. L. Bioinspired Design of Dynamic Materials. *Adv. Mater.* **21**, 2361–2374 (2009).
- Ehrbar, M., Schoenmakers, R., Christen, E. H., Fussenegger, M. & Weber, W. Drug-sensing hydrogels for the inducible release of biopharmaceuticals. *Nat Mater* **7**, 800–804 (2008).
- Kampf, M. M. *et al.* A Gene Therapy Technology-Based Biomaterial for the Trigger-Inducible Release of Biopharmaceuticals in Mice. *Adv Funct Mater* **20**, 2534–2538 (2010).
- Christen, E. H. *et al.* Conditional DNA-Protein Interactions Confer Stimulus-Sensing Properties to Biohybrid Materials. *Adv Funct Mater* **21**, 2861–2867 (2011).
- Geraths, C., Daoud-El Baba, M., Charpin-El Hamri, G. & Weber, W. A biohybrid hydrogel for the urate-responsive release of urate oxidase. *J. Control. Release* (2013).
- Jakobus, K., Wend, S. & Weber, W. Synthetic mammalian gene networks as a blueprint for the design of interactive biohybrid materials. *Chemical Society reviews* **41**, 1000–1018 (2012).
- Gubeli, R. J., Ehrbar, M., Fussenegger, M., Friedrich, C. & Weber, W. Synthesis and Characterization of PEG-Based Drug-Responsive Biohybrid Hydrogels. *Macromolecular rapid communications* **33**, 1280–1285 (2012).
- Andre, F. E. & Safary, A. Summary of clinical findings on Engerix-B, a genetically engineered yeast derived hepatitis B vaccine. *Postgraduate medical journal* **63 Suppl 2**, 169–177 (1987).
- Bachmann, M. F. & Jennings, G. T. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat Rev Immunol* **10**, 787–796 (2010).
- Miller, A. K., Verwey, W. F. & West, M. K. A laboratory evaluation of the chemotherapeutic properties of cathomycin. *Antibiotics annual* **3**, 924–928 (1955).
- Solotorovsky, M., Valiant, M. E., Frost, B. M. & Cuckler, A. C. Further studies on microbiological activity of novobiocin. *Antibiot and Chemother* **8**, 86–92 (1958).
- Hahm, H. A. *et al.* Novobiocin in combination with high-dose chemotherapy for the treatment of advanced breast cancer: a phase 2 study. *Biology of blood and marrow transplantation: journal of the American Society for Blood and Marrow Transplantation* **6**, 335–343 (2000).
- Eder, J. P., Wheeler, C. A., Teicher, B. A. & Schnipper, L. E. A phase I clinical trial of novobiocin, a modulator of alkylating agent cytotoxicity. *Cancer research* **51**, 510–513 (1991).
- Jalota, S., Bhaduri, S. B. & Tas, A. C. Using a synthetic body-fluid (SBF) solution of 27 mM HCO₃[−] to make bone substitutes more osteointegrative. *Mat Sci Eng C-Bio S* **28**, 129–140 (2008).
- Schulze, A., Schieck, A., Ni, Y., Mier, W. & Urban, S. Fine Mapping of Pre-S Sequence Requirements for Hepatitis B Virus Large Envelope Protein-Mediated Receptor Interaction. *J Virol* **84**, 1989–2000 (2010).
- Deepen, R., Heermann, K. H., Uy, A., Thomssen, R. & Gerlich, W. H. Assay of Pres Epitopes and Pres1 Antibody in Hepatitis-B Virus Carriers and Immune Persons. *Med Microbiol Immun* **179**, 49–60 (1990).
- Glebe, D. *et al.* Pre-S1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* **77**, 9511–9521 (2003).
- Ali, O. A., Huebsch, N., Cao, L., Dranoff, G. & Mooney, D. J. Infection-mimicking materials to program dendritic cells in situ. *Nat Mater* **8**, 151–158 (2009).
- Singh, A., Suri, S. & Roy, K. In-situ crosslinking hydrogels for combinatorial delivery of chemokines and siRNA-DNA carrying microparticles to dendritic cells. *Biomaterials* **30**, 5187–5200 (2009).
- Farra, R. *et al.* First-in-Human Testing of a Wirelessly Controlled Drug Delivery Microchip. *Science Translational Medicine* **4** (2012).
- Weber, C. C. *et al.* Broad-spectrum protein biosensors for class-specific detection of antibiotics. *Biotechnology and bioengineering* **89**, 9–17 (2005).
- Gripon, P. *et al.* Infection of a human hepatoma cell line by hepatitis B virus. *P Natl Acad Sci USA* **99**, 15655–15660 (2002).
- Ladner, S. K. *et al.* Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: A novel system for screening potential inhibitors of HBV replication. *Antimicrob Agents Ch* **41**, 1715–1720 (1997).

Acknowledgements

We would like to thank Clément Mouchereud for assistance with the animal experiments, Stefan Seitz for the production of the HBV stocks, Carina Gillig and Christian Friedrich for support with the rheological characterization, Davide Corti (Humabs, Bellinzona, Switzerland) for providing the humanized antibody MA 18/7 and Benjamin Ritter for assistance with the NMR measurements. This work was supported by the European Research Council under the European Community's Seventh Framework Programme (FP7/2007–2013)/ERC Grant agreement n° 324622-SmartVaccines, the Excellence Initiative of the German Federal and State Governments (EXC-294 and GSC-4), the INTERREG IV Upper Rhine project No. A20 and the Deutsche Krebshilfe (K.S. and S.U. grant number 109883).

Author contributions

R.J.G. performed the hydrogel synthesis, R.J.G. and D.H. analyzed the immune response, K.S. performed the neutralization experiments, G.C.E.H. and M.D.E.B. planned and performed the mice experiments, R.J.G., M.E., S.U. and W.W. planned the study and analyzed the data. R.J.G. and W.W. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: A patent application covering the hydrogel design of which R.J.G. and W.W. are inventors has been filed by the University of Freiburg.

How to cite this article: Gubeli, R.J. *et al.* Pharmacologically Triggered Hydrogel for Scheduling Hepatitis B Vaccine Administration. *Sci. Rep.* **3**, 2610; DOI:10.1038/srep02610 (2013).



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