Immunogenic compositions assembled from tobacco mosaic virus-generated spherical particle platforms and foreign antigens

Olga Karpova,¹ Nikolai Nikitin,¹ Sergey Chirkov,¹ Ekaterina Trifonova,¹ Anna Sheveleva,¹ Ekaterina Lazareva¹ and Joseph Atabekov¹,²

¹Department of Virology, Moscow State University, Moscow 119991, Russia
²A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119991, Russia

We reported recently that RNA-free spherical particles (SPs) generated by thermal remodelling of tobacco mosaic virus (TMV) are capable of binding GFP to their surface. Here, we show that SPs represent a universal particle platform that can form compositions by binding a diversity of various foreign proteins/epitopes of viral and non-viral origin to their surface. Numerous molecules of a foreign protein linked to the SP surface were revealed by immunogold electron microscopy. Several SP-based compositions were obtained containing one of the following foreign antigens: antigenic determinant A of rubella virus E1 glycoprotein; a recombinant protein containing the M2e epitope of influenza virus A protein M2; a recombinant antigen consisting of three epitopes of influenza virus A haemagglutinin; potato virus X (PVX) coat protein (CP); BSA; and PVX CP fused with the epitope of plum pox virus CP. The ‘mixed’ compositions could be also assembled by binding two different foreign antigens to each of the SPs. Immunogenicity of foreign antigens adsorbed or linked covalently to SPs in the SP-based compositions was examined. The antigenic specificity of foreign antigens was retained, whereas their immunogenicity increased significantly. It was inferred that SPs exhibit immunopotentiating activity, in particular in the form of compositions comprising SP and foreign antigen linked covalently to their surface by formaldehyde.

INTRODUCTION

Tobacco mosaic virus (TMV) particles are rod-like (18 × 300 nm) and consist of identical protein subunits packed closely by hydrophobic bonds into a rigid tube (Zaitlin & Israel, 1975; Butler, 1999; Klug, 1999).

We have reported recently that spherical nanoparticles (SPs) generated by thermal remodelling of native TMV are composed of viral coat protein (CP) subunits denatured and assembled specifically into SPs (Atabekov et al., 2011). Apparently, under conditions of thermal denaturation, the TMV CP subunits acquire a unique conformation favourable for their assembly into SPs. The size of the SPs varies over a wide range and does not correlate with the size of the SP generated by an original TMV rod (SP monomer, 53 nm). The SPs consist of thermally denatured TMV CP molecules and do not contain RNA, are water-insoluble, and are heterogeneous in size but uniform in shape. The size of SPs depends on virus concentration and varies from nanoparticles (diameter 53–150 nm) to microparticles (diameter up to 800 nm and more). We have also reported that TMV-generated SPs are capable of forming compositions with GFP molecules bound to the SP surface (Atabekov et al., 2011). The SPs have no structural analogues and represent a new type of biogenic particle nanoplatform.

The particular aims of the present study were: (i) closer examination of binding specificity of foreign proteins/epitopes to TMV-generated SP platforms and (ii) determination of immunogenic activity of foreign antigens linked to the surface of the SP-based compositions.

RESULTS

In vitro assembly of compositions comprising the SP platform and entire foreign protein molecules linked to the SP surface

The assembly procedure of the SP-based compositions involves a short incubation of the SPs with a protein of interest (foreign protein) required for binding to the SP surface. Apparently, the adsorption of a foreign protein to the SP surface is based on non-covalent (e.g. electrostatic and hydrophobic) bonds. Then, the SP–protein/epitope complexes are separated from unbound protein and,
finally, the complex is treated by formaldehyde to link a foreign antigen covalently to the SPs. The foreign antigen/epitope binding to the SPs was detected by fluorescence microscopy, immunofluorescence microscopy and immunoelectron microscopy with gold-labelled secondary antibodies, illustrated schematically in Fig. 1.

We reported recently that SPs can bind entire protein molecules of GFP (Mₐ of 30 kDa) (Atabekov et al., 2011). In the present study, evidence is provided that the surface of SPs has a unique adsorption capacity, being capable of binding a diversity of various proteins/epitopes.

In particular, this feature of SPs is illustrated by an experiment where the SPs were incubated with FITC-labelled potato virus X (PVX) CP (CPFITC) molecules (Mₐ of 25 kDa). Samples containing 50 µg SPs and 20 µg fluorescent PVX CPFITC were incubated at room temperature for 10 min and centrifuged at 2000 g to separate the SP–PVX CPFITC complexes from unbound CPFITC. The supernatant was analysed at 495 nm (wavelength of maximum absorption by FITC). It was found that >50% of FITC-labelled PVX CP was adsorbed by SPs. The fluorescence microscopy data indicated that all of the SPs used for PVX CPFITC binding were covered with fluorescent molecules (Fig. 2a, b). In separate experiments, evidence is provided that, in contrast to SPs, no CPFITC could be bound to native TMV particles (data not shown).

Next, fluorescence microscopy showed that SPs were decorated successfully by fluorescent molecules of BSA (Mₐ of 66 kDa) (Fig. 2c), supporting the conclusion that entire molecules of various proteins can be linked to the surface of SPs.

Another model antigen, the recombinant protein N1 (Mₐ of 26 kDa), which represents a fusion product of N-deleted PVX CP with epitope SMLNPIFTPA from plum pox virus (PPV) CP (PPV N1 protein), was applied for SP-based composition assembly. The PPV N1 protein was visualized at the SP surface by primary antibodies to PPV and secondary antibodies conjugated with fluorophore Alexa Fluor 555. Immunofluorescence microscopy (Fig. 2d) showed that entire PPV N1 protein molecules were linked to the surface of the SP platform.

Characteristics of the association of SPs and foreign antigen molecules

In a series of experiments, the salt stability of the SP-based compositions was examined. The results presented in Fig. 3(a) show that SP–CPFITC complexes treated by formaldehyde are stable in the range 0.05–0.40 M NaCl. Conversely, the SP–CPFITC complexes not treated by formaldehyde decayed to yield CPFITC in 0.05–0.10 M NaCl (Fig. 3b). Similar results were obtained with the SP–BSAFITC complexes. Therefore, our results suggest that electrostatic interactions play a certain role in SP–PVX CPFITC and SP–BSAFITC assembly. It is noteworthy that the isoelectric point of the SPs is about 3.7–3.9 (Nikitin et al., 2011), whereas the pI values of PVX CP and BSA are 6.73 and 4.6–4.8, respectively.

Furthermore, the structure of SP–PVX CP complexes stabilized by formaldehyde was characterized by Western

![Fig. 1](http://vir.sgmjournals.org) 401

**Fig. 1.** (a) Schematic representation of detection of foreign protein molecules on the SP-based compositions. (b) Detection by fluorescence microscopy of FITC-conjugated proteins linked to the SP platform. (c) Detection of proteins/antigens linked to the SP platform by immunofluorescence microscopy (with primary and fluorophore-conjugated secondary antibodies) or by immunoelectron microscopy with gold-labelled secondary antibodies.

![Fig. 2](http://vir.sgmjournals.org) 401

**Fig. 2.** (a–c) Fluorescence microscopy of compositions comprising SPs with foreign proteins linked to their surface. (a) PVX CP labelled with FITC linked to the SPs (SP–PVX CPFITC). (b) Control: the same image presented in phase contrast. (c) BSA labelled with FITC linked to SPs (SP–BSA FITC). (d) Immunofluorescence microscopy of PPV N1 protein linked to the SP surface and revealed by primary antibodies to PPV and secondary antibodies conjugated with fluorophore. Bars, 3 µm.
blotting. Individual protein bands were revealed in control SP and PVX CP preparations [Fig. 4(a, b), lanes 1 and 2, respectively]. In contrast, multiple bands were produced by formaldehyde-treated SP–PVX CP compositions [Fig. 4(a, b), lanes 3]. The present study was not focused on the precise identification of the proteins that constituted each of the multiple bands on the Western blots. It was most significant that the majority of the large-size components reacted with antibodies to both the PVX CP and SPs [Fig. 4(a, b), lanes 3]. Conceivably, the larger products represented multimeric combinations of the SP-derived protein molecules linked to the PVX CP molecules by formaldehyde. However, the most rapidly migrating components in Fig. 4(a, b) (lanes 3) reacted selectively with antisera to SPs and to PVX CP respectively, i.e. might correspond to dimers (or multimers) of the SP-derived and PVX CP molecules.

In order to examine more closely the location of the foreign antigen molecules in the SP–foreign protein complexes, immunoelectron microscopy with gold-labelled secondary antibodies (immunogold microscopy) was used. Fig. 4(c, d) shows that numerous PVX CP molecules were bound to the surface of the SP–CP compositions.

**Immunofluorescence microscopy of SP–antigen/epitope compositions**

In addition to the model proteins mentioned above (GFP, PVX CP, BSA and PPV N1 protein), several recombinant epitopes potentially attractive for vaccine development were utilized for SP-based composition assembly. The compositions assembled from the SP platform and foreign antigens linked to their surface are shown in Fig. 5: the segments (65–212 aa) containing three epitopes of haemagglutinin (HA) of human influenza virus A (Fig. 5a, b); tetratetrapeptides of antigenic determinant A of rubella virus E1 glycoprotein (Fig. 5c); the M2e epitope, representing the N-terminal 23 aa of human influenza virus A membrane protein M2 (Fig. 5d). The sequence of M2e is conserved in all influenza virus A isolates (Lamb et al., 1985; Fiers et al., 2004). Therefore, the N-terminal M2e could be attractive for nanovaccine development. In the present work, the M2e epitope was fused to dehydrofolate reductase (DHFR).
Furthermore, the possibility of concurrent binding of more than one antigen to the SP surface was examined. To this end, two antigens (PVX CP and DHFR–M2e) were used simultaneously for SP-based composition assembly. Two different fluorophores were used to discriminate between PVX CP and DHFR–M2e bound to the surface of the same SP. Fig. 6(b, c) shows that both of these antigens were revealed on the surface of each SP in the sample.

**Immunogenic properties of the SP-based compositions**

The immunogenicity of foreign antigens linked to or mixed with SP-based compositions was examined. In a series of experiments, the SP–PVX CP complexes representing PVX CP linked to the surface of SPs by formaldehyde were used to immunize mice. In control immunizations, PVX CP alone or a mixture of SPs with PVX CP was used. The titres of antiserum obtained by immunization with PVX CP mixed with or linked to SPs were about 1/140,000, whereas those of antiserum induced by PVX CP alone were about 1/10,000 (Fig. 7a). This result provides evidence to suggest that SPs exhibit immunopotentiating ability enhancing the humoral immune response. There was very little difference in stimulation of the immune response by PVX CP mixed with SPs or linked to the SP surface (Fig. 7a, Exp. 1). In a similar independent experiment (Fig. 7a, Exp. 2), the difference between the levels of enhancement by the SP + PVX CP mixture and SP–PVX CP linked composition was somewhat more pronounced. Also, it is noteworthy that the booster efficiency of SP–PVX CP compositions and that of complete Freund’s adjuvant (the strongest adjuvant until recently) were reasonably comparable to each other. The antiserum titres were about 1/100,000 and 1/240,000, respectively (Fig. 7a).

Again, the titres obtained by immunization with SP–PVX CP compositions were higher by a factor of ten than that elicited by PVX CP alone. A considerable difference in immunogenicity was observed in experiments where a simple mixture (SPs + PPV N1 protein) was compared with formaldehyde-treated SP–PPV N1 compositions (Fig. 7b). Comparison of the data presented in Fig. 7(a, b) suggests that the efficiency of immune-response stimulation by SP-based compositions could depend on the specific properties of the foreign antigen (PVX CP or PPV N1) used. It should be mentioned that the results obtained did not depend on the size of the SPs (100 or 500 nm) used in the experiments outlined above.

**DISCUSSION**

A kinetic unit in TMV solution is a compact nanoparticle containing a certain amount of bound water. The degree of
widely different molecular size (proteins and epitopes (Figs 2 and 5). Foreign proteins of and the capacity to bind a diversity of functionally unrelated structural transitions impart some unique properties to but favourable for assembly into SPs. Apparently, the foreign protein subunits makes them water-insoluble, CP (Caspar, 1963). The thermal denaturation and misfold-
hydration can vary and depends on the properties of viral CP (Caspar, 1963). The thermal denaturation and misfold-
ing of TMV protein subunits makes them water-insoluble, but favourable for assembly into SPs. Apparently, the structural transitions impart some unique properties to the TMV CP molecules thermally denatured and assembled into SPs, including very high stability (Atabekov et al., 2011) and the capacity to bind a diversity of functionally unrelated proteins and epitopes (Figs 2 and 5). Foreign proteins of widely different molecular size (Mr in the range 16–66 kDa) can be adsorbed and linked to the surface of SPs.

We found that SP–CPFITC and SP–BSAFITC complexes treated by formaldehyde were stable in the range 0.05–0.40 M NaCl, whereas complexes not treated by formalde-
hyde were disrupted in 0.05–0.10 M NaCl. These results suggest that electrostatic interactions play a certain role in SP–protein complex assembly. We have reported previously (Atabekov et al., 2011) that thermal denaturation of native TMV protein subunits results in production of water-
insoluble SPs. Apparently, assembly from denatured protein subunits makes the SP surface substantially hydrophobic. The precise role of different bonds in SP assembly is unknown; however, it seems reasonable to suggest that hydrophobic interactions are also involved in SP–foreign antigen binding.

Particularly noteworthy is that multiple components of varying size were revealed by Western blot analyses of formaldehyde-treated (SP–PVX CP) compositions. Our data provide evidence that complexes of varying size, comprising SP–CP, CP–CP and SP–derived protein mole-
cules linked covalently together, were produced after SP stabilization by formaldehyde.

The foreign protein molecules linked to the SP surface could not be revealed by electron microscopy with negative staining or by atomic force microscopy, but were readily visualized by immunogold electron microscopy (Fig. 4c, d). It should be noted that the exact structural organization of PVX CP subunits bound to the SP surface is obscure. There is little likelihood that individual CP subunits linked to SPs are revealed in Fig. 4(c, d). We have reported previously (Kaftanova et al., 1975; Atabekov et al., 2007) that PVX CP exists in a monomeric form (S20, about 2S) only at extreme conditions (0.5 % SDS, 0.25–1.0 M CaCl2), whereas in water, pH 6.8, two aggregates with sedimentation coefficients of 3–5S and 10–15S are present. Therefore, it is most probable that aggregates of PVX CP subunits were revealed by immunogold electron microscopy. Fig. 4(c, d) shows that numerous PVX CP molecules are exposed on the SP surface, not as random agglomerates, but with reasonable regularity. It can be presumed that PVX CP bound to some adsorption sites on the SP platform.

Furthermore, the SP platforms were shown to be capable of being assembled in vitro into ternary complexes comprising the SP and two types of antigen exposed on its surface (Fig. 6). This observation supports the hypothesis that poly-
valent antisera could be produced in this way.
Demonstrating the induction of immune responses is a key step in developing new vaccines. Immunofluorescence microscopy and immunogold electron microscopy showed that foreign proteins/epitopes linked covalently to the surface of the SP platform were readily recognized by relevant primary antibodies. These results indicate that the antigenic specificity of proteins/epitopes was not changed after their binding to the SP surface.

The results presented in Fig. 7 indicate that the titres of serum antibodies to two viral proteins (PVX CP and PPV N1) increased dramatically when these proteins were mixed with the SPs prior to immunization. This effect was most pronounced when foreign antigen was bound covalently to the SP surface by formaldehyde. To our knowledge, this is the first report of stimulation of the humoral immune response to foreign proteins by the particle platforms generated by thermal remodelling of a native virus. In the present paper, the nature of the immune response was not studied in detail; however, the data obtained suggest the induction of Th2 cells, promoting antibody-based immunity. Therefore, the SPs could be considered as an adjuvant functioning either by presenting antigens or as some immune potentiator, or a combination of both (Heegaard et al., 2011).

Taken together, our data show that SPs represent a new type of universal particle platform that can be assembled in vitro into immunogenic compositions by binding various foreign antigens to their surface. Therefore, these SP-based compositions could be regarded as candidates for in vitro nanovaccine assembly.

The implementation of SP-based immunogenic compositions carrying antigen linked to their surface could have several advantages over attenuated, chemically inactivated and DNA vaccines. (i) Application of immunogenic compositions assembled in vitro rules out the possibility of reversion to pathogenic forms because SP-based compositions are assembled from genetically inert components; compositions assembled in vitro are safe for humans, as plants and animals have no common pathogens. (ii) Production of SPs is inexpensive, as the yield of TMV may reach 10 g (kg fresh leaves) and the procedure of purification is simple (Zaitlin & Israel, 1975); no sterilization of SP platforms is required, as they are produced by heating TMV at 94–98 °C. The procedure of in vitro assembly of immunogenic compositions from preformed components (SP platforms and antigens) is fast (takes about 1 h). (iii) The SP platforms are capable of enhancing the immune response of the animal to foreign antigens.

On the whole, fast, simple and widely available technologies for stable, biologically safe SP platform production and for in vitro assembly of SP-based immunogenic compositions have been developed.

**METHODS**

**Virus purification.** The TMV U1 strain was isolated from systemically infected *Nicotiana tabacum* L. cv. Samsun plants as described by Novikov & Atabekov (1970).

**Generation of SPs.** Heating of TMV at the concentration of 0.1 or 1 mg ml⁻¹ was performed in a Tercyc thermocycler (DNA Technology) for 10 s at 94–98 °C and SPs of two major sizes (100 or 500 nm) were obtained (Atabekov et al., 2011).

**SP–foreign antigen composition formation.** Generally, 50 µg SPs was incubated with 1–2 µg foreign antigen/epitope in water at room temperature for 10 min. A maximum amount of foreign protein bound to the SP surface was 10 µg per 50 µg particles. At this stage, SP–foreign antigen complex formation occurred due to electrostatic and/or hydrophobic interactions. Then, the generated complexes were centrifuged at 2000 g to remove unbound antigen. The pellet consisting of SP–foreign antigen complex was resuspended in water and then stabilized with 0.05% formaldehyde (10 min at room temperature). The excess formaldehyde was removed by centrifugation of SP–foreign antigen compositions at 2000 g.

**Compositions comprising SPs and FITC-labelled PVX CPFITC and BSAFITC.** PVX CP (25 kDa) was isolated from purified PVX as described previously (Atabekov et al., 2000). FITC (Sigma) was added to 2 mg PVX CP ml⁻¹ according to the manufacturer’s protocol with some modifications. The protein–FITC solution was dialysed first against PBS to remove the unbound label and then exhaustively against water. The FITC-labelled PVX CP was used for SP–PVX CPFITC composition production. To produce FITC-labelled PVX CP, 50 µg SPs was incubated with 1 µg PVX CPFITC in water. Thereafter, the complex was stabilized by 0.05% formaldehyde (10 min at room temperature). Production of SP–CPFITC was detected by fluorescence microscopy. A similar procedure was used to obtain BSAFITC. The 66.3 kDa BSA fraction V (MP Biomedical) was utilized.

**Western blot analysis.** Western blotting was performed as reported previously (Karpova et al., 2006).

**Bacterially expressed proteins/epitopes.** All recombinant DNA procedures were carried out by standard methods (Sambrook et al., 1989). Recombinant constructs expressing fusion (His)₆ proteins were generated by cloning the PCR-amplified fragments into the pQE plasmid vector (Qiagen). Restriction fragments were ligated into the corresponding sites of the expression vector as described by Ivanov et al. (1994). All other methods used were in accordance with Sambrook et al. (1989). The purification of (His)₆-tagged proteins from cultures followed a general procedure described by the manufacturer (Qiagen) for denaturing Ni-NTA chromatography.

The following bacterially expressed recombinant antigens/epitopes were used for binding to the surface of SP platforms:

(i) Antigenic determinant A of rubella virus E1 glycoprotein (Hobman et al., 1997). The recombinant protein of about 21–22 kDa was constructed on the base pQE-BT-4A and contained six His residues at the N terminus, and four repeats of E1 glycoprotein domain A (32 aa × 4).

(ii) A recombinant protein of 26 kDa consisting of DHFR fused to the 23 aa long M2e epitope of influenza virus A protein M2 (Fan et al., 2004). DHFR served as a carrier for fusion with the M2e epitope (DHFR–M2e). The ability of anti-M2 mAbs to reduce virus replication implicates the M2 protein and, in particular, M2e as a vaccine target (Tompkins et al., 2007).

(iii) A recombinant protein of 16 kDa consisting of three neutralizing epitopes (aa 65–212) of the human influenza virus A HA protein. Design of HA polypeptides was based on the HA precursor gene (A/Kurgan/5/05).

(iv) The N-terminal 20 aa were deleted from PVX CP, and the epitope of PPV CP (10 residues) (Fernández-Fernández et al., 2002).
was fused to PVX CP. The resulting chimeric protein N1 of 26 kDa contained epitope SMLNPITPA at the N terminus of PVX CP.

Mouse antisera against bacterially expressed HA polyepitopes, DHFR–M2e and tetrapeptipeptide A of rubella virus protein E1 were obtained as described by Erkohna et al. (2000). Rabbit antisera to PVX and PPV were obtained by intracutaneous animal immunization with purified preparation of viruses.

**SP–foreign antigen/epitope composition detection**

**Fluorescence microscopy.** The foreign antigens/epitopes bound to the SPs were detected by the immunofluorescence method. For immunostaining, the SP–foreign antigen/epitope complexes were adsorbed for 5 min at room temperature onto Formvar-coated coverslips. After incubation, the samples were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with water for 5 min. The samples were then preincubated for 30 min with 1% BSA, 0.05% Tween 20 in PBS (7 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl) and then incubated for 30 min at room temperature in a humid chamber with mouse or rabbit primary antibodies against the foreign antigen/epitope in PBS supplemented with 1% BSA and 0.05% Tween 20. In control experiments, the samples were incubated without primary antibodies. After incubation, the samples were washed three times with washing buffer composed of PBS supplemented with 0.25% BSA and 0.05% Tween 20. Then, primary antibodies bound to antigens were revealed using secondary chicken anti-mouse antibodies conjugated with the fluorophore Alexa Fluor 488 (Invitrogen) or TRET (Sigma), or secondary donkey anti-rabbit antibodies conjugated with the fluorophore Alexa Fluor 555 (Invitrogen) (30 min at room temperature). After binding, the samples were washed three times with washing buffer and once with PBS. Then, all samples were stained with DABCO (1,4-diazabicyclo-[2.2.2]octane; Sigma) anti-fading mounting medium for 30 min at 4 °C. The results of immunostaining were analysed using an Axiovert 200M fluorescence microscope (Carl Zeiss) equipped with a digital cooled camera ORCAII-ERG2 (Hamamatsu) and using AxioVision software (Carl Zeiss Imaging Solutions).

**Immuno-gold electron microscopy.** The SP–PVX CP complexes stabilized by formaldehyde were adsorbed onto Formvar film attached to 200-mesh nickel electron microscopy grids. The specimens were blocked by 1% BSA in PBS for 20 min and incubated with polyclonal antibodies to PVX CP. Samples were washed with PBS and secondary gold-conjugated antibodies were added for 20 min. The gold particles were 12 nm in diameter. After immunolabelling, the grids were washed with distilled water and air-dried. The specimens were stabilized by formaldehyde were adsorbed onto Formvar film attached to 200-mesh nickel electron microscopy grids. The specimens were 12 nm in diameter. After immunolabelling, the grids were washed with distilled water and air-dried. The specimens were examined under a transmission electron microscope LEO-912AB (LEO).

**Immunogenicity of the SP-based compositions.** Groups of 6–8-week-old white female mice (wild type), each containing five animals, were immunized with PVX CP alone or mixed either with SPs or an equal volume of complete Freund’s adjuvant (Difco), or with PVX CP linked to the SP surface. The animals were given three intraperitoneal injections at intervals of 2 weeks. A dose of 20 μg PVX CP and 500 μg SPs (or 20 μg PVX CP alone) in 0.4 ml sterile water was used for immunization. The animals were bled 1 week after the last injection. The same immunization scheme was employed using PPV N1 protein as an antigen. The titres of antisera were determined by indirect ELISA using PVX or PPV as capture antigens, pools of mouse antisera to PVX CP and PPV N1 protein, respectively, as primary antibodies, and horseradish peroxidase-labelled anti-mouse IgG W402B (Promega) as detection antibodies. A pool of non-immune murine sera was used as a negative control. The titre of antiserum was expressed as a value of its dilution, giving an A505 twice that of the negative control. Animal handling and maintenance were performed according to the RF Law about Animal Protection from Abuse (1 December 1999) and the RF Veterinary Law (22 August 2004). All experimental protocols were approved by the Lomonosov Moscow State University Animal Protection Committee.

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