Published in final edited form as:

Adv Mater. 2015 July 15; 27(27): 4081–4086. doi:10.1002/adma.201501603.

Step-index Optical Fiber Made of Biocompatible Hydrogels

Prof. Myunghwan Choi,

Harvard Medical School and Wellman Center for Photomedicine, Massachusetts General Hospital, 65 Landsdowne St, UP-5, Cambridge, Massachusetts 02139, USA; Global Biomedical Engineering, Sungkyunkwan University, Center for Neuroscience and Imaging Research, Institute for Basic Science, 2066, Seobu-ro, Jangn-Gu, Suwon-Si, Gyeong Gi-Do, South Korea

Dr. Matjaž Humar,

Harvard Medical School and Wellman Center for Photomedicine, Massachusetts General Hospital, 65 Landsdowne St, UP-5, Cambridge, Massachusetts 02139, USA; Condensed Matter Department, J. Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia

Mr. Seonghoon Kim, and

Harvard Medical School and Wellman Center for Photomedicine, Massachusetts General Hospital, 65 Landsdowne St, UP-5, Cambridge, Massachusetts 02139, USA; Graduate School of Nanoscience and Technology, Korea Advanced Institute of Science and Technology, 291 Daehak-Ro, Yusong-Gu, Daejeon 305-701, Korea

Prof. Seok-Hyun Yun*

Harvard Medical School and Wellman Center for Photomedicine, Massachusetts General Hospital, 65 Landsdowne St, UP-5, Cambridge, Massachusetts 02139, USA

Keywords

optical fiber; hydrogel; biocompatible; implantable; sensing

The limited penetration of light into biological tissue poses a formidable challenge in many applications of light in biomedicine. Although a few approaches, such as near-infrared spectral window^[1] and wave-front shaping^[2], have shown to improve light penetration, optical waveguides, particularly optical fibers, represent the current gold-standard solution in clinical uses^[3]. However, most light-guiding systems are based on conventional solid-state optical materials, such as glasses and inorganic plastics, which unfortunately are not compatible with the biological system^[4]. To overcome this limitation, there have been continuing efforts to develop optical waveguides using biocompatible materials, such as polyethylene glycol (PEG)^[5], silk^[6], agarose gel^[7], and even bacteria^[8]. However, the light-guiding structures demonstrated to date have not yet been considered for biomedical applications because of the following limitations. First, their light-guiding efficiency is not sufficient to deliver light over an organ-scale distance, which is over 10 cm for humans. The $1/e$ attenuation lengths of the waveguides ranged from only tens of microns^[8] to a few centimeters^[5]. Equally importantly, the previous approaches used a single material with

* syun@hms.harvard.edu.

uniform refractive index. In such core-only waveguides, guiding is achieved by total internal reflection at the interface between the material and the surrounding medium. As a result, the guiding efficiency is considerably degraded when the waveguides are introduced into living biological tissues, where the local refractive index in the tissue (1.33-1.51) in contact with the core is comparable to or higher than that of the core material^[9].

In this communication, we demonstrate a new type of optical fibers that solve these technical limitations. First, we employ a core-clad structure with an overall step index profile, which enable us to confine light within a core surrounded by a clad layer. We demonstrate excellent guiding efficiency and stability within living biological tissues. Second, we use biocompatible hydrogels for the core and clad, for the first time to our knowledge. Besides their desirable mechanical flexibility, the hydrogels allow us to incorporate various functional fluorophores and nanoparticles into their porous structure to build various types of specialty fibers for biomedical applications including *in vivo* optical sensing and light-induced therapy.

For low-loss light guiding, the core and clad must have high optical transparency, and the core should have higher refractive index than the cladding. To meet these basic requirements, we investigated two widely-used biocompatible hydrogels: PEG and alginate^[10]. We have previously reported that the optical properties of PEG hydrogels were highly dependent on precursor concentration^[5]. At concentrations of PEG-diacrylate (PEGDA, 700 Da) higher than 15%, the optical transparency of PEG hydrogels after UV-induced polymerization increased with the monomer concentration (Figure 1a). The measured refractive index (n) of the hydrogels was in good agreement with calculated values by a linear weighted sum of the refractive indices of constituent materials (PEGDA, $n=1.46$; water, $n=1.331$), increasing linearly with the precursor concentration (Figure 1b). The PEG hydrogels showed slightly higher refractive indices than the precursor solutions due to shrinkage during photo-polymerization. At low precursor concentrations of alginate (1-4% wt vol⁻¹), the optical transparency of alginate hydrogels decreased with the concentration (Figure 1c). The refractive index of alginate hydrogels increases as a linear function of precursor concentration and was close to that of water ($n=1.331$) due to their high water content (Figure 1d). Considering both the refractive index and transparency, we decided to use 80-90% wt vol⁻¹ PEG hydrogels for the core and 1-2% wt vol⁻¹ alginate hydrogels for the cladding.

We developed a two-step process to fabricate the core and cladding (Figure 2a). First, the core was fabricated by using a platinum-cured silicone tube as a mold. The inner diameter of the tube mold determined the diameter of the core (Figure 2b). Precursor solution for PEG hydrogel was injected into the tube and photocrosslinked by exposure to ultraviolet light. After the mold was swollen in dichloromethane for 30 min, the core was extracted. Then, the core was dipped in a sodium alginate and calcium chloride solution, typically 2–4 times until multi-layered alginate cladding is formed to a desired thickness (typically 100–150 μm). The thickness of the each clad layer was controlled by the temperature of the dipping solution. Thinner clads are formed at higher temperature due lower viscosity of the solution. It typically took about 2 hour to complete the entire process. This fabrication process was reproducible and scalable (Figure 2c).

We evaluated the light guiding property of fabricated hydrogel fibers. Laser light at a wavelength of 492 nm was coupled to a hydrogel fiber, and the side-scattering pattern of the light transmitting along the fiber was imaged when the fiber was placed in air (Figure 2d) or embedded between thin porcine tissue slices (Figure 2e). From the axial intensity profile of side-scattered light, the propagation loss of the hydrogel fiber at 492 nm was measured to be $0.32 \pm 0.02 \text{ dB cm}^{-1}$ in air and $0.42 \pm 0.01 \text{ dB cm}^{-1}$ in tissue (Figure 2f). The slightly lower loss in air is presumably due to the contribution by light leaked from the core (due to defects) but guided farther through the clad-air interface. By contrast, a single-index core-only PEG hydrogel fiber fabricated without alginate coating showed a significantly higher loss of 1.15 dB cm^{-1} in tissue. In terms of $1/e$ penetration depth, the step-index hydrogel fibers offer light guidance over 10 cm in the visible spectrum (Figure 2g).

The high permeability of hydrogels allowed us to incorporate functional molecules into the fibers (Figure 3a). The pore size of a PEG hydrogel made with 700 Da monomers is approximately 1.5 nm, which permit small molecules to penetrate into the hydrogels by diffusion^[11]. Dye molecules, such as rhodamine 6G, were easily loaded into the core by dipping the distal end of the core in dye solution prior to cladding encapsulation (Figure 3b). The incorporated dye absorbs coupled excitation light, adding additional attenuation to the fiber. The absorptive attenuation is linearly proportional to concentration and extinction coefficient. In case of 1 μM rhodamine 6G, the fiber attenuation at the absorption peak (530 nm) is estimated to be $\sim 0.1 \text{ dB cm}^{-1}$ but it becomes negligible in the spectral region other than the absorption band (e.g. 0.002 dB cm^{-1} at 600 nm). This diffusion-based solution doping process does not involve any chemical reactions and is therefore reversible. For example, photo-bleached dyes could be removed from the fibers by washing, and active dyes could be replenished.

Alternatively, more robust functionalization by covalent bonding is also possible by incorporating complementary reactive functional groups. We encapsulated avidin into the core, and the fabricated fiber was doped by dipping it in a solution containing biotin-conjugated fluorophores. As an example, we doped a fiber with three different biotin-conjugated fluorophores, Atto 488, Atto 520, and Atto 565, respectively, in three distinct positions along the fiber (Figure 3c). This was achieved by applying 1 μl dye droplets to the fiber. When blue laser light (491 nm) was coupled into the fiber, it emitted bright fluorescence at distinct spectra from the dye-doped regions (Figure 3c).

Molecules larger than the pore size can be physically entrapped by mixing them in the precursor solution before crosslinking. Using this method, we embedded gold nanoparticles (GNPs) with a diameter of 50 nm and, therefore, a plasmonic resonance-enhanced absorption peak at a wavelength of 532 nm. When the GNP-doped fiber was pumped with continuous-wave 532 nm laser light, significant heat was generated from GNP's and the temperature of the fiber increased by $\sim 16^\circ\text{C}$ in 1 min with a coupled optical power of 0.6 W (Figure 3d). At the same pumping condition, a control fiber without GNPs showed a much less temperature increase of $\sim 3^\circ\text{C}$ (Figure 3e). This result demonstrates the potential of the hydrogel fiber for photothermal applications.

We next explored the possibility of using a dye-doped hydrogel fiber for optical amplification. We loaded rhodamine 6G in the core of a fiber using the diffusion method described above. For optical pumping, a Q-switched laser light was illuminated to a 5-mm segment of the fiber by focusing through a cylindrical lens (Figure 4a). The output emission from the fiber tip was collected through an objective lens and analyzed by a spectrometer with a cooled charge coupled detector (CCD). At pump fluences less than $5 \mu\text{J}/\text{mm}^2$, the typical fluorescence emission of rhodamine 6G with a spectral width of $\sim 50 \text{ nm}$ was measured (Figure 4b,c). As the pump intensity increased, the emission power increased superlinearly, accompanying narrowing of spectral width down to 6 nm in full-width-half-maximum (FWHM) (Figure 4b,c). This phenomena, known as amplified spontaneous emission, results from the amplification of guided fluorescence light along the fiber^[12].

Another mode of light amplification was observed in the tangential direction of the fiber through whispering gallery mode (WGM) guiding^[13]. To generate WGM lasing, we arranged the optical geometry so that the direction of pumping and collection are the same in the transverse plane of the fiber (Figure 4d). At pump intensities above threshold, sharp emission spectral peaks appeared at wavelengths of $\sim 585 \text{ nm}$ (Figure 4e). The output energy increased superlinearly with a distinct threshold at $\sim 80 \mu\text{J}/\text{mm}^2$ (Figure 4f). Camera images showed predominant light extraction at the core-clad interface above laser threshold, as expected from bidirectional (clockwise and counter clockwise) WGM oscillations (Figure 4f, inset). Below threshold, fluorescence was emitted uniformly from the entire core. Lasing was suppressed when we intentionally disrupted the WGM path by cutting the fiber to a D-shape. These results collectively suggest WGM lasing.

Potential medical applications of the hydrogel fibers include deep-tissue light-based therapies based on photothermal or photodynamic therapy^[14]. The high flexibility of hydrogel fibers allows them to be implanted and integrated in tissues easily or inserted through natural opening, such as the gastrointestinal tracts. We tested this feasibility in live mice. A hydrogel fiber was inserted into the intestine through the rectum (Figure 5A), which was not possible with a conventional silica optical fiber because of its stiffness. Laparotomy confirmed efficient delivery of light to the distal end despite the relatively small bending radius of the fiber.

We also tested the feasibility of optical sensing of blood oxygenation levels by absorbance spectroscopy. Two hydrogel fibers were implanted in subcutaneous tissues of an anesthetized mouse with their tips separated by 5 mm from each other. One of the fibers delivered excitation light at 560 nm and 640 nm into the tissue, and the other fiber collected the light diffused through the tissue (Figure 5b). As the oxygen level of the supplied gas was modulated by switching between nitrogen and oxygen, the changes in the optical intensity signal at 560 nm and 640 nm were measured and, using Beer Lambert law, converted to relative oxy- and deoxy-hemoglobin concentrations. As expected, inhalation of nitrogen caused sudden drop in oxygen tension in the blood, i.e. a decrease in oxy-hemoglobin and an increase in deoxy-hemoglobin. The oxygen tension was reversed by inhalation of oxygen (Figure 5c).

Although our current fibers successfully achieved organ-scale transmission (> 10 cm), the performance can be further improved. The primary cause of loss is thought to be the surface roughness (~ 1 μm) of the tube mold. Improved mold surface would lower the waveguide loss significantly. Besides the step-index structure, other types, such as a double clad guiding structure or even a gradient index profile, should be achievable by using more than two materials or a solution doping technique. Furthermore, it may be possible to fabricate fibers with much smaller core diameters for single-mode operation. Much thinner sizes at even submicron scale may be feasible by microfluidics or electrospinning techniques^[15]. Injectable hydrogel, such as thermoresponsive sol-gel hydrogels, may allow minimally invasive implantation through a needle by forming a hydrogel fiber structure *in situ*^[16]. Formation of complex microstructured fibers *in situ* may also be feasible by introducing advanced microfluidic techniques on the needle. Furthermore, hydrogels with highly stretchable or self-healing properties could be adapted to improve mechanical stability^[17].

In summary, we have described the fabrication, optical characteristics, and applications of core-clad step-index hydrogel optical fibers. Low-loss light guiding (< 0.42 dB/cm) over the entire visible spectrum was achieved *in vivo*. The hydrogel core allowed various functional materials, such as organic molecules and plasmonic nanoparticles, to be incorporated for the generation of fluorescence, amplified spontaneous emission and WGM lasing, as well as photothermal heat generation. We expect that the biocompatible, functional hydrogels fibers will have broad impact in the field of photomedicine by bringing photonic tools towards *in vivo* system.

Experimental Section

Fabrication of core-clad fiber

Platinum-cured silicone tubes (Cole Parmer) with inner diameters of 250–1000 μm were used as a mold for the core. Precursor solution composed of 80% wt vol⁻¹ PEGDA (700 Da; Sigma Aldrich), 5% wt vol⁻¹ 2-hydroxy-2-methyl-propiophenone (Sigma Aldrich) in distilled water was injected in the tube through a syringe adapted with a syringe filter with 0.45 μm pore. The PEG hydrogel was formed by photocrosslinking the solution with exposure to UV (365 nm, 5 mW cm⁻²; Spectroline) for 5 min. The tube with the crosslinked core was immersed in dichloromethane for 30 min, and then the core was isolated from the swollen tube. The core was immersed in distilled water at least for 1 hour to remove unreacted chemicals. To form the clad layer, the core was immersed in alginate solution (2 % wt vol⁻¹; Sigma Aldrich) and then in calcium chloride solution (100 mM; Sigma Aldrich). This procedure was repeated to form a multi-layer clad. Successful fabrication of the core-clad fiber was checked by phase-contrast microscopy (Olympus).

Optical characterization

Refractive indices of hydrogels were measured with a digital refractometer (Sper Scientific). Hydrogels were prepared in a standard 1-cm-wide poly(methyl methacrylate) disposable cuvettes, and optical attenuation was measured using a scanning spectrophotometer over a spectral range from 250 to 1000 nm (Thermo Scientific). To make homogeneous alginate gels in a cuvette, sodium alginate (1–4 % wt vol⁻¹; Sigma Aldrich) was slowly gelated with

combination of CaCO_3 (15 mM; Sigma Aldrich) and δ -gluconolactone (15 mM; Sigma Aldrich) as described previously^[18].

Optical setup for optical amplification measurement

For dye doping, the fiber core with diameter of 800 μm was immersed in rhodamine-6G solution (0.1% wt vol⁻¹) for over 12 hours, and then the alginate clad was added by dip-coating. The fiber was mounted on a slide glass and placed on a 3-axis micrometer. Laser pulses from optical parametric oscillator (Quanta Ray MOPO-700, Spectra Physics; 535 nm, 5 ns, 10 Hz) were illuminated to the fiber from the side for optical pumping, and the output emission from the fiber was collected through an objective lens and analyzed with a spectrometer (Andor, 300 mm focal length).

Animal experiments

8- to 12-week-old BALB/c nude mice (Jackson Laboratory) were used after anesthetized by intraperitoneal injection of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹). For the experiment demonstrating implanted light source, the descending colon of the mouse was flushed several times with warm saline and the fiber was introduced through the rectum^[19]. Abdominal laparotomy was followed to gain visual access to the descending colon where fiber tip was placed. For reflectance oximetry, two fibers were subcutaneously implanted, and oxygen and nitrogen was alternately ventilated with an interval of 30 to 60 s. The change in reflectance at 560 nm and 640 nm in wavelength was measured respectively. The relative change in intensity, $\Delta I/I$, was converted to oxy- and deoxy-hemoglobin levels as previous described^[20]. In brief, attenuation for each wavelength was represented as linear summation of absorptions by oxy- and deoxy-hemoglobin using Beer-Lambert law, and concentration for each hemoglobin type was decomposed by solving the set of linear equations. All animal experiments were performed in compliance with institutional guidelines and approved by the subcommittee on research animal care at the Harvard Medical School.

Acknowledgements

We thank Prof. Xiangwei Zhao for discussions. This work was funded by the U.S. National Institutes of Health (P41EB015903, R21EB013761) and Marie Curie International Outgoing Fellowship N°627274 within the 7th European Community Framework Programme.

References

- [1]. Hong G, Diao S, Chang J, Antaris AL, Chen C, Zhang B, Zhao S, Atochin DN, Huang PL, Andreasson KI. Nature Photon. 2014; 8:723. Choi M, Choi K, Ryu S-W, Lee J, Choi C. J. Biomed. Opt. 2011; 16:046008. [PubMed: 21529077]
- [2]. Ji N, Sato TR, Betzig E. Proc. Natl. Acad. Sci. USA. 2012; 109:22. [PubMed: 22190489]
- [3]. Keiser G, Xiong F, Cui Y, Shum PP. J. Biomed. Opt. 2014; 19:080902. [PubMed: 25166470] Gora MJ, Sauk JS, Carruth RW, Gallagher KA, Suter MJ, Nishioka NS, Kava LE, Rosenberg M, Bouma BE, Tearney GJ. Nat. Med. 2013; 19:238. [PubMed: 23314056]
- [4]. Nizamoglu S, Gather MC, Yun SH. Adv. Mater. 2013
- [5]. Choi M, Choi JW, Kim S, Nizamoglu S, Hahn SK, Yun SH. Nature Photon. 2013; 7:987.
- [6]. Parker ST, Domachuk P, Amsden J, Bressner J, Lewis JA, Kaplan DL, Omenetto FG. Adv. Mater. 2009; 21:2411.

- [7]. Jain A, Yang AH, Erickson D. Opt. Lett. 2012; 37:1472. [PubMed: 22555708]
- [8]. Xin H, Li Y, Liu X, Li B. Nano Lett. 2013; 13:3408. [PubMed: 23786313]
- [9]. Jacques SL. Phys. Med. Biol. 2013; 58:R37. [PubMed: 23666068]
- [10]. Hoffman AS. Adv. Drug Del. Rev. 2002; 54:3.
- [11]. Cruise GM, Scharp DS, Hubbell JA. Biomaterials. 1998; 19:1287. [PubMed: 9720892]
- [12]. Yang P, Wirnsberger G, Huang HC, Cordero SR, McGehee MD, Scott B, Deng T, Whitesides GM, Chmelka BF, Buratto SK. Science. 2000; 287:465. [PubMed: 10642543]
- [13]. Humar M, Ravnik M, Pajk S, Muševič I. Nature Photon. 2009; 3:595. McCall S, Levi A, Slusher R, Pearton S, Logan R. Appl. Phys. Lett. 1992; 60:289.
- [14]. Bagley AF, Hill S, Rogers GS, Bhatia SN. ACS nano. 2013; 7:8089. [PubMed: 23961973]
- [15]. Yarin AL, Zussman E, Wendorff J, Greiner A. J. Mater. Chem. 2007; 17:2585.
- [16]. Choi CH, Yi H, Hwang S, Weitz DA, Lee CS. Lab Chip. 2011; 11:1477. [PubMed: 21390381]
- Jeong B, Kim SW, Bae YH. Adv. Drug Del. Rev. 2002; 54:37.
- [17]. Sun J-Y, Zhao X, Illeperuma WR, Chaudhuri O, Oh KH, Mooney DJ, Vlassak JJ, Suo Z. Nature. 2012; 489:133. [PubMed: 22955625] Phadke A, Zhang C, Arman B, Hsu C-C, Mashelkar RA, Lele AK, Tauber MJ, Arya G, Varghese S. Proc. Natl. Acad. Sci. USA. 2012; 109:4383. [PubMed: 22392977]
- [18]. Kuo CK, Ma PX. Biomaterials. 2001; 22:511. [PubMed: 11219714]
- [19]. Choi M, Yun SH. Opt. Express. 2013; 21:30842. [PubMed: 24514657]
- [20]. Suh M, Bahar S, Mehta AD, Schwartz TH. NeuroImage. 2006; 31:66. [PubMed: 16480899]

Figure 1.

Optical properties of bulk hydrogels in cuvettes. (a, b) Measured attenuation coefficients, (a), and refractive indices, (b), of PEG hydrogels made with a monomer size of 700 Da at concentrations of 15-90% w/v. (c, d) Absorption spectra, (c), and refractive indices, (d), of alginate hydrogels at concentrations of 1-4% w/v.

Figure 2.

Fabrication of core-clad fibers. (a) Fabrication steps. Step 1, PEG hydrogel is formed by photocrosslinking in a tube mold. Step 2, the core is extracted from the tube by swelling the tube in dichloromethane. Step 3, the alginate hydrogel clad layer is added by dip-coating. Step 3 can be repeated to obtain a desired clad thickness. (b) Phase-contrast images of four hydrogel fibers with different sizes, immersed in distilled water. Arrows indicate the outer clad-water interface and arrowheads indicate interfaces between alginate layers formed by multiple dip coating. The numbers on top represent the thickness of the core and the clad in μm (i.e. 250/60 indicates 250- μm core diameter and 60- μm clad thickness; the outer diameter is thus 370 μm). The third image (550/200) shows a four-layer cladding. (c) A photograph of a 1-meter-long fiber. (d) Light guidance in air. Blue laser light (492 nm) was coupled to a hydrogel fiber (800/100) through a 10 $\times$ objective lens (obj). (e) Light guidance of a fiber (800/100) sandwiched between two thin porcine tissue slices. Scale bar, 1 cm. (f) Propagation loss of three different fibers. (g) Corresponding 1/e propagation distances of the bare (800/0) and shelled (800/100) fibers.

Figure 3.

Functionalized hydrogel fibers. (a) A schematic for functionalization of the core. Small-molecular fluorophores can be loaded through passive diffusion. Immobilization of the fluorophores can be achieved by introducing reactive groups (e.g. avidin) in the core. Larger objects, such as gold nanoparticles (GNP) can be physically trapped during the fabrication of the hydrogel core. (b) A fluorescence image of a dye-doped fiber. Rhodamine-6G was loaded in the fiber by dipping the tip in a solution of the fluorophore. When blue laser was coupled, the dye-doped region emits yellow fluorescence from excited rhodamine-6G molecules. (c) A fluorescence image of a fiber doped with three different fluorophores, Atto 488, 520 and 565, respectively, along the fiber. The biotin-conjugated fluorophores were incorporated into the avidin-encapsulated core. (d-e) Photothermal operation of a GNP-doped fiber. Green laser (532 nm, 800 mW) was coupled to the fiber to induce photothermal heating. Temperature was measured using a thermocouple in contact with the fiber.

Figure 4.

Light amplification in a dye-doped fiber. The core was doped with rhodamine-6G and pumped with a Q-switched laser at 535 nm. (a) A setup for amplified spontaneous emission (ASE). Approximately 5 mm length of the fiber was pumped, and the guided ASE output at the end of the fiber was analyzed. (b) Output spectra measured at two different pump levels. (c) Output energy (red) and spectral width (blue) as a function of pump fluence. (d) A setup for whispering-gallery-mode (WGM) lasing. The red ring illustrates the optical paths of the laser modes oscillating along the circumferential interface between the core and the cladding. (e) Output spectra measured at three different pump levels. (f) The output power curve showed a distinct lasing threshold about 80 $\mu\text{J}/\text{mm}^2$. Inset, a side-view image of the fiber above laser threshold. The red region indicates the laser light that was leaked from the fiber by scattering and recorded in a camera.

Figure 5.

Demonstration of uses *in vivo*. (a) Pictures of a mouse with a hydrogel fiber administered to the colon through the rectum. The emission of red laser (640 nm) at the distal end of the fiber is seen through the skin and confirmed by abdominal laparotomy. (b-c) Reflectance oximetry of tissues. (b) Two hydrogel fibers—one for excitation and the other for collection—were implanted subcutaneously in an anesthetized mouse to measure the intrinsic optical absorption signal in response to oxygen/nitrogen ventilation. (c) Typical time-lapse traces of the calculated concentrations of oxy-hemoglobin (HbO), deoxy-hemoglobin (HbR), and total hemoglobin (HbT= HbO + HbR).